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FOREWORD

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Introduction

Cyclin E is an important regulator of the coordinated cell cycle, as it is rate limiting for the G1/S phase transition. Deranged cyclin E expression has been found both quantitatively (overexpression) and qualitatively (multiple isoforms) in almost all breast cancer cell lines and patient tumor tissue examined to date. Disruption of such an important cell cycle protein could potentially contribute to transformation and/or tumorigenicity in breast cancer, but the direct involvement of cyclin E in breast cancer has yet to be elucidated. Intracellular antibodies, or intrabodies, are a powerful tool for evaluating the *in vivo* function of a given protein. Single-chain antibodies (sFvs), consisting of the heavy and light chain variable regions of an IgG linked by a flexible peptide spacer, are able to fold and retain the antigen binding specificity of the parental antibody. SFvs can be modified for targeted expression to the cytosol, nucleus, or endoplasmic reticulum (ER), where they can bind to and inactivate their target antigen, thus generating a "phenotypic knockout" of the protein of interest. In this study we have constructed two anti-cyclin E sFvs and generated constructs that successfully target their expression as intrabodies to the cytosol or nucleus of breast cancer cells. We propose to use breast cancer cell lines stably or inducibly expressing these anti-cyclin E intrabodies to evaluate their effect on the nuclear trafficking and associated kinase activity of cyclin E. We will then evaluate the biological effects (i.e. doubling time, cell cycle distribution, growth in soft agar, tumorigenicity in nude mice) of anti-cyclin E intrabody expression in these cell lines. This study should provide direct evidence to determine the functional significance of cyclin E in the abnormal growth and transformation of breast cancer, as well as determine if there is merit in cyclin E targeted strategies for the treatment of breast cancer.

Annual Summary

The first aim of this proposal was to generate anti-cyclin E single chain antibodies (sFvs) based on the parental anti-cyclin E monoclonal antibody producing hybridoma cell lines HE-12 and HE-172. These hybridomas were generated and characterized in the lab of Dr. Edward Harlow at Harvard University. We obtained these hybridomas and grew them in culture for the purpose of isolating poly-adenylated messenger RNA (mRNA) from log phase cells using the Micro-FastTrack mRNA Isolation Kit (Invitrogen). Using this mRNA as template, we then performed RT-PCR to amplify the approximately 350 base pair cDNA fragments which encode the heavy (V_H) and light (V_K) chain variable regions of the anti-cyclin E antibodies (Fig 1). RT-PCR was performed using degenerate primers designed against the Framework 1 (5') and Framework 4 (3') regions of the IgG heavy and light chain genes. These primers incorporated HindIII (5') and XbaI (3') restriction sites to facilitate cloning into a sequencing vector. Several clones were sequenced and the resulting nucleotide and deduced amino acid sequences were subjected to GeneBank query to ensure they encoded for unique peptide sequences. Unique heavy and light chain cDNAs were PCR amplified to add overlapping oligonucleotides encoding a (Gly₄Ser)₃ linker, and the two fragments were then linked by an additional overlap extension PCR into sFvs in the order of V_H -Linker- V_K (Fig 1).

Using this procedure, we have successfully cloned the heavy and light chain variable region genes of both the HE-12 and HE-172 hybridomas, and linked them into sFvs. The nucleotide and deduced amino acid sequences for these sFv constructs are depicted in Appendix A. The first construct to be completed was that from the HE-172 cell line, and we initially cloned the HE-172 construct into a baculovirus expression system for expression and purification of sFvs in Sf9 insect cells, and assessment of cyclin E binding affinity by Western blot or ELISA. Although we have successfully expressed epitope tagged (influenza Hemagglutinin Antigen-HA) HE-172 sFv in insect cells (Figure 2), purification of sufficient protein for binding analysis has not been achieved using the baculovirus system. We have recently completed cloning of both intrabody constructs into a mammalian expression vector (pRc/CMV) with the goal of producing

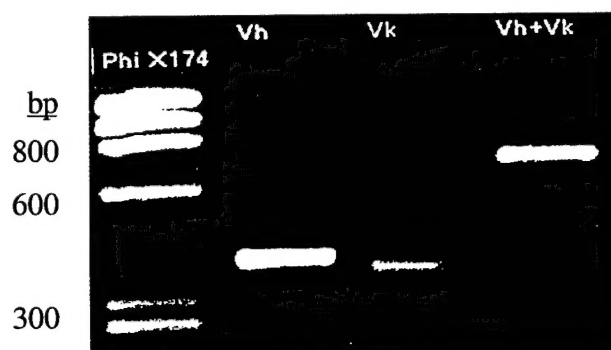


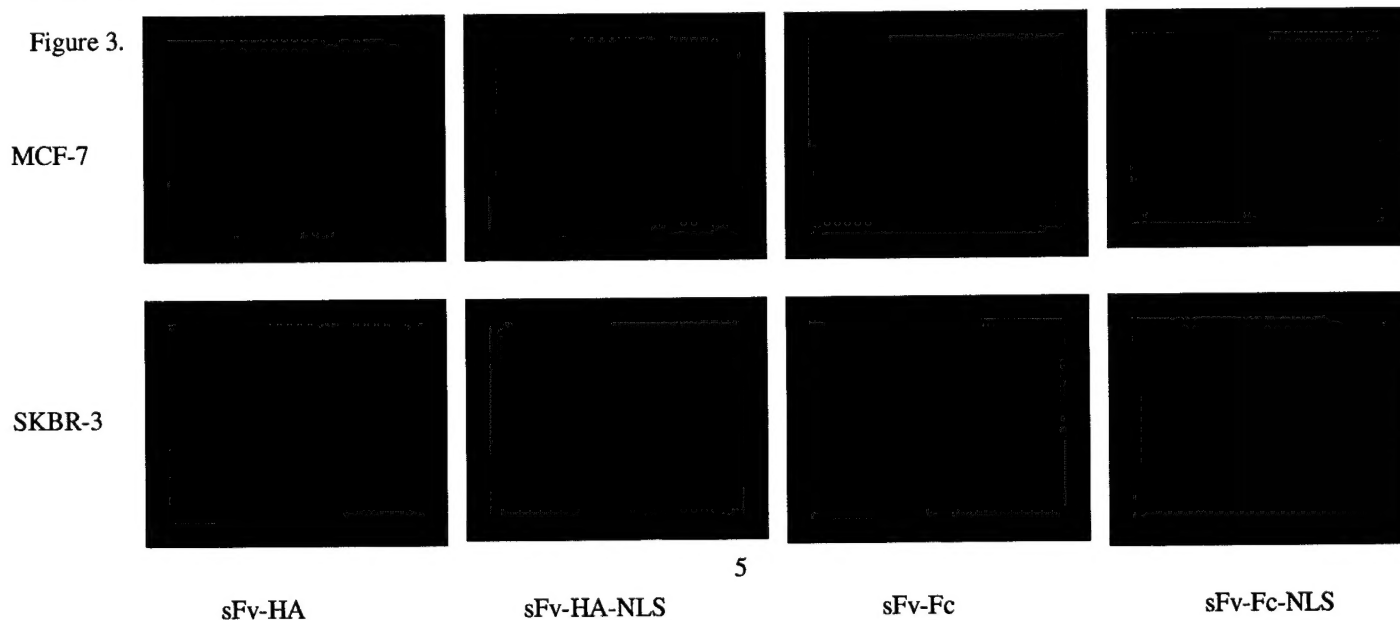
Figure 1

secreted sFv-Fc fusion proteins by cloning the sFv constructs in frame with the cDNA encoding the human IgG Fc fragment. Addition of the Fc fragment has been shown to increase protein stability, enhances sFv secretion, and facilitates purification of the sFv-Fc fusion proteins by Protein A affinity chromatography. The IgG heavy chain signal sequence was added 3' to the sFv-Fc constructs to target them to the secretory pathway. These constructs are currently being evaluated in COS-1 cells.

Figure 2. Expression of HE-172 sFv-HA in Sf9 insect cells. **A.** Sf9 cells infected with recombinant baculovirus carrying the HE-172 sFv-HA construct (left) or mock infected cells (right) were fixed and stained with anti-HA FITC antibody 48 hrs post-transfection. **B.** Supernatants from infected or mock infected Sf9 cells were separated by SDS-PAGE, transferred to nitrocellulose, blocked, blotted with rabbit anti-HA (1°), and anti-Rabbit-HRP (2°) antibodies, and developed using ECL detection kit (Amersham).



The second aim of this proposal was to generate mammalian expression vectors for use in the targeted expression of anti-cyclin E intrabodies to the cytosol or nucleus of the breast cancer cell lines MCF-7 and SKBR-3. A schematic representation of the constructs for cytosolic and nuclear expression of sFv and sFv-Fc intrabodies is shown in Appendix B. All of the constructs depicted have been completed for the HE-172 sFv, and were used in transient transfection assays using MCF-7 and SKBR-3 cells. The sFv was modified by addition of the HA epitope tag for use in immunodetection. Additional sFv-Fc constructs were made to assess the effect of the Fc fragment on expression, stability, and targeting of sFvs in breast cancer cells. Antibodies specific for the human Fc fragment were used for immunodetection of sFv-Fc constructs. Addition of the SV40 nuclear localization signal (NLS) to the 3' end of the sFv or sFv-Fc construct was used to target expression of the protein to the nuclear compartment. Figure 3 presents immunofluorescence results from transient transfection of MCF-7 and SKBR-3 cells. Briefly, MCF-7 or SKBR-3 cells were grown in 6-well plates and transfected with the indicated constructs using GenePorter transfection reagent (Gene Therapy Systems). 48 hours post-transfection, cells were fixed and stained with either anti-HA-FITC or anti-human Fc-FITC antibody. These results indicated the ability to successfully target expression of sFv and sFv-Fc intrabodies to the cytosol or nucleus of both MCF-7 and SKBR-3 cells. In addition, the fluorescence intensity seen in these experiments supported the notion that intrabody stability was increased by either 1) targeting expression of the sFv to the nucleus, thereby removing the protein from the harsh cytosolic environment (sFv vs sFv-NLS), or 2) addition of the Fc fragment, thereby increasing cytosolic (sFv vs sFv-Fc) as well as nuclear compartment (sFv-NLS vs sFv-Fc-NLS) stability.



Key Research Accomplishments

- Completion of HE-12 and HE-172 single-chain antibody cloning
- Completion of HE-12 and HE-172 sFv-Fc mammalian expression vectors for use in expression, purification, and binding analysis of sFv-Fc proteins manufactured in a mammalian cell line
- Completion of HE-172 mammalian expression vectors for targeted cytosolic and nuclear targeting of sFv and sFv-Fc constructs
- We have shown sFvs can be expressed and successfully targeted to the cytosol and nucleus of the breast cancer cell lines MCF-7 and SKBR-3

Reportable Outcomes

There have been no publications, patents, licences, degrees earned, etc resulting from this funding as of this date. An abstract of this research was presented at the Era of Hope Breast Cancer Research Program Meeting in Atlanta, GA, June 8-12, 2000.

Conclusions

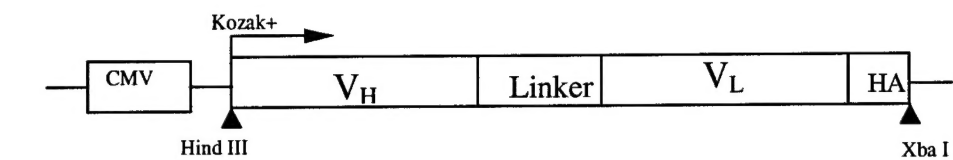
Intrabodies provide a powerful tool to examine the in vivo function of a given protein. We are using an intrabody approach to investigate the role aberrant cyclin E expression plays in breast cancer transformation and tumorigenesis by attempting to phenotypically knock out cyclin E. Toward this goal, we have generated two anti-cyclin E single chain antibodies, and displayed their ability to be expressed and targeted to the cytosol and nucleus of breast cancer cells. We are currently evaluating the binding affinity of the anti-cyclin E intrabodies, and will attempt to generate breast cancer cell lines stably or inducibly expressing these intrabodies. We will then evaluate the biological effects of intrabody expression on cyclin E activity in an attempt to elucidate the specific role cyclin E plays in the tumorigenicity and proliferation of breast cancer.

Appendix A. HE-12 and HE-172 sFv Nucleotide and Deduced Amino Acid Sequences

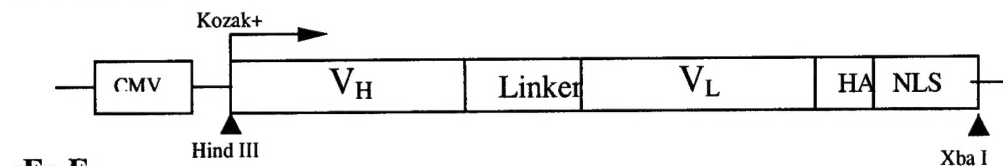
	10	20	30	40	50	
172SFV.SEQ	1 GAGGTGCAGC	TGGAGGAGTC	AGGACCTGAA	CTGAAGAAGC	CTGGAGAGAC	50
HE12SFV.SEQ	1	T.....TG	50
	60	70	80	90	100	
172SFV.SEQ	51 AGTCAGGATC	TCCTGCAAGA	CTTCTGGGTA	TAAGTTCACA	ACTGCTGGAA	100
HE12SFV.SEQ	51	A.....GCAGTA	100
	110	120	130	140	150	
172SFV.SEQ	101 TGCAGTGGGT	GCAAAAGATG	CCAGGAAAGG	GTTTGAAGTG	GATTGGCTGG	150
HE12SFV.SEQ	101 ..A.C.....	A..C..GCTAA....T	CT.G.....	150
	160	170	180	190	200	
172SFV.SEQ	151 ATAAACACCC	ACTCTGGAGT	GCCAAAATAT	GCAGAAGACT	TCAAGGGACG	200
HE12SFV.SEQ	151	A.....ACT..T....	..G.....	200
	210	220	230	240	250	
172SFV.SEQ	201 GTTTGCCTTC	TCTTTGGAAG	CCTCTGCCAG	CACTGTATAT	TTACAGATAA	250
HE12SFV.SEQ	201 ...G.....ACC...	..G.....C.	250
	260	270	280	290	300	
172SFV.SEQ	251 ACAACCTCAA	AGATGAGGAC	ACGGCTACGT	ATTCTGTAC	GAGAAATAAA	300
HE12SFV.SEQ	251 ...G.....	A.....TAGT	C-----C.T	300
	310	320	330	340	350	
172SFV.SEQ	301 TATAGGTACG	AGGCCTGGTT	TGCTTACTGG	GGCCAAGGGA	CTCTGGTCAC	350
HE12SFV.SEQ	301	G-----	-----T..	350
	360	370	380	390	400	
172SFV.SEQ	351 TGTCTCTGCA	GGTGGCGGTG	GCTCCGGAGG	TGGTGGGAGC	GGTGGCGGCG	400
HE12SFV.SEQ	351	400
	410	420	430	440	450	
172SFV.SEQ	401 GATCTGACAT	TGTGCTCACA	CAGTCGGCAG	CAATCATGTC	TGCATCTCCA	450
HE12SFV.SEQ	401G...C..C	TC.CTT....	G.TGA.CATT	450
	460	470	480	490	500	
172SFV.SEQ	451 GGGGAGAGGG	TCACCGTGTC	CTGCA-GT--	-GCCAG----	-CTCAAGT--	500
HE12SFV.SEQ	451 ..AC..ACCA.	C.T..A.C..	T....A..CA	A.T...AGCC	T..T.GA..AG	500
	510	520	530	540	550	
172SFV.SEQ	501 -----GTAAG	TTACATTT--	ACTGGTACCA	GCAGAAGCCA	GGATCCTCCC	550
HE12SFV.SEQ	501 TGATG.CG..	AC.T....GA	.T....TGTT	A....G....	..CCAG..T.	550
	560	570	580	590	600	
172SFV.SEQ	551 CCAAACCTCG	GATTTATCGC	ACATTCAACC	TGGCTTCTGG	AGTCCCTGCT	600
HE12SFV.SEQ	551 ..A..G.G..CT	A..C....TG	GTG..CT..G.AC.....AC	600
	610	620	630	640	650	
172SFV.SEQ	601 CGCTTCAGTG	GCACTGGGTC	TGGGACCTCT	TACTCTCTCA	CAATCAGCAG	650
HE12SFV.SEQ	601 A.G....C..A..	A....AGA.	.TTA.A..G.	A.....	650
	660	670	680	690	700	
172SFV.SEQ	651 CATGGAGGCA	GAAGATGCTG	CCACTTATTA	CTGCCAGCAG	TATCATA-GT	700
HE12SFV.SEQ	651 AG.....T	..G...TTG.	GAGT.....	T...TG...A	-G.T.C.CA.	700
	710	720	730	740	750	
172SFV.SEQ	701 TATCCAGTCA	CGTTCGGTGC	TGGGACCAAG	CTGGAGATCA	AACGT.....	750
HE12SFV.SEQ	701 .T...TCAG.G	A..C.....	750

	10	20	30	40	50	
HE12SFV.AMI	1 EVQLVESGPE	LKKPGETVKI	SCKASGYTFT	KYGMNWKQA	PGKSLKFLGW	50
HE172SFV.AMI	1E.....R.	...T...N..	TA..Q..QKM	...G..WI..	50
	60	70	80	90	100	
HE12SFV.AMI	51 INTYTGEPTY	ADDFEGRLAF	SLETSANTAY	LQINSLKNED	MATYFCV---	100
HE172SFV.AMI	51 ...HS.V.K.	E..K..F..S.V.N..D..	T.....TRNK	100
	110	120	130	140	150	
HE12SFV.AMI	101 ----SLLRYW	GQGTLLTVSA	GGGGSGGGGS	GGGSDIVLT	QSPLTSLVTI	150
HE172SFV.AMI	101 YRYEAWFA..AAIM.ASP	150
	160	170	180	190	200	
HE12SFV.AMI	151 GQPASISCKS	SQSLDSGDGE	TYLNWLLQRP	GQSPKRLIYL	VSKLDSGVDP	200
HE172SFV.AMI	151 .ERVTV..SA	.S.V-----	S.IY.YQ.K.	.S...PW..R	TFN.A....A	200
	210	220	230	240	250	
HE12SFV.AMI	201 RFTGSGSGTD	FTLKISRVEA	EDLGYYCQWQ	VTHFPQTGGG	GTKLEIKR..	250
HE172SFV.AMI	201 ..S.....S	YS.T..SM..	..AAT...Q.	YHSY.V...A	250

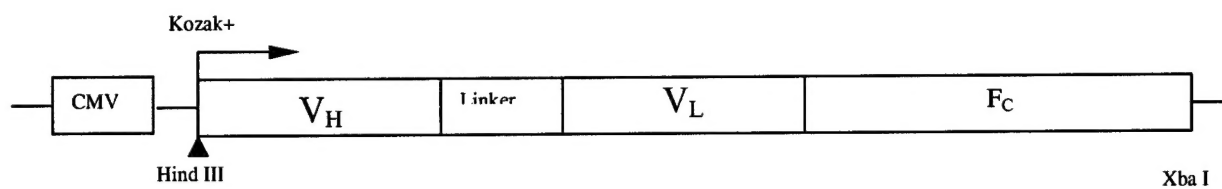
Appendix B. Schematic Representation of Anti-cyclin E sFv and sFv-Fc Constructs.



sFv-HA-NLS



sFv-F_C



sFv-F_C-NLS

